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<p>(54) Title: NOVEL CELL SYSTEMS HAVING SPECIFIC INTERACTION OF PEPTIDE BINDING PAIRS</p> <p>(57) Abstract</p> <p>This invention relates to novel modified host cells which express heterologous fused proteins and methods of screening for test samples having peptide-binding activity; wherein the modified host cell comprises: (a) a gene sequence encoding a heterologous fusion protein; said fusion protein comprising a first peptide of a peptide binding pair, or segment of said first peptide, which is joined to either a DNA binding domain or its corresponding transcriptional activation domain of a transcriptional activation protein; (b) a gene sequence encoding a heterologous fusion protein, said fusion protein comprising a second peptide of the peptide binding pair in (a), or a segment thereof, fused to either a DNA binding domain or its corresponding transcriptional activation domain, whichever one is not employed in (a); (c) a reporter gene operatively associated with the transcriptional activation protein, or a portion thereof; (d) optionally, a deletion or mutation in the chromosomal DNA of the host cell for the transcriptional activation protein if present in the selected host cell.</p>			
<p>The diagram illustrates a cell with various internal components. At the top left, there are two circular structures labeled "BD RECEPTOR". To the right of these are two elongated, striped structures labeled "BD". Below these, there are two circular structures labeled "AD RECEPTOR". To the right of these are two elongated, striped structures labeled "AD". At the bottom center, there is a circular structure labeled "CUP LIGAND". To the right of this is a small rectangular box labeled "UASG". Further to the right, there is another small rectangular box labeled "GAL1-HIS". A line connects the "UASG" and "GAL1-HIS" boxes. At the bottom right, the word "CELL" is written.</p>			

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**Novel Cell Systems Having Specific Interaction
of Peptide Binding Pairs**

Field of the Invention

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This invention relates to novel cells which express heterologous fused proteins and methods of screening for compounds having peptide-binding activity; wherein the methods employ the novel cells of this
10 invention.

Background of the Invention

The specific binding of a pair of peptides to each other triggers a vast number of functions in a living cell. For example, the specific binding of a ligand to a surface receptor serves as the trigger for cellular responses to many external signals. In mammals, cells respond to a wide variety of circulating peptide hormones, often through single transmembrane domain receptors. It is certainly recognized that the cytokine receptor superfamily illustrates the diverse aspects of cellular function, and physiological responses. Recent examinations of cytokine receptor function have revealed differing ligand-receptor protein stoichiometries including both 2-protein (ligand/receptor) (Cunningham et al., 1991; Staten et al., 1993) and 3-protein (ligand/receptor/receptor or ligand/receptor/transducer) interactions (Young, 1992; Taga and Kishimoto, 1992; Mui and Miyajima, 1994). The intricacies of such protein associations have been investigated using *in vitro*, often laborious, methods (Fuh et al., 1992; 1993; Davis et al., 1993) since genetically malleable expression systems have been
20 unavailable. The present invention is directed to novel modified host systems which can be used for such protein
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investigations, yet the novel systems which significantly less laborious.

Recently reported systems in the art refer to a "2-hybrid" system as discussed by Fields and Song, 1989 and also Chein et al., 1991. The "2-hybrid" system involves differential interactions between the separable DNA binding and activation domains of the yeast transcriptional activator, Gal4. Heterologous proteins are expressed as hybrid proteins fused to either half of Gal4 (see Figure 1; Fields and Song, 1989; Chein et al., 1991 for discussion of the 2-hybrid system). The productive interaction of the heterologous proteins brings the two halves of the Gal4 protein in close proximity, activating expression of a scorable reporter gene. To this date, such 2-hybrid systems have been disclosed as useful for determining whether a first given test peptide sequence has binding activity for the known sequence of second peptide; wherein the affinity of the test peptide for the known peptide is unknown. Studies using such a system have been directed to analyzing intracellular proteins such as transcription factors and kinase-target protein interactions (Yang et al., 1992; Durfee et al., 1993; Li et al., 1994).

The novel modified cells of this invention and novel methods incorporating these cells provide significant advancement for the study and discovery of peptide mimics, including ligand mimics and receptor mimics. At this time no one has developed an efficient and specific screening system to investigate these areas. By employing in the cell a peptide binding pair for which the binding affinity is known, the present invention permits the investigation of peptide binding pairs, such as a ligand and receptor, wherein the peptides bind via extracellular interactions. The present invention creates exponential advantages for the discovery of

compounds which can interact as ligands for specific receptors or transducers. Potential ligands include, but are not limited to, mammalian hormones with the receptors being a cognate extracellular ligand-binding peptide.

5 Furthermore, the present invention describes the use of cell systems which express multiple heterologous proteins, including the two heterologous fused proteins to establish the specific and reversible binding of the ligand and receptor. The specific interaction of the

10 above-described binding is readily detected by a measurable change in cellular phenotype, e.g. growth on selective medium.

Summary of the Invention

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One aspect of the present invention relates to the novel modified host cells for the expression of heterologous fusion proteins. The novel modified host cells comprise:

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a) a gene sequence encoding a heterologous fusion protein; said fusion protein comprising a first peptide of a peptide binding pair, or segment of said first peptide, which is joined to either a DNA binding domain or its corresponding transcriptional activation

25 domain of a transcriptional activation protein;

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b) a gene sequence encoding a heterologous fusion protein, said fusion protein comprising a second peptide of the peptide binding pair in (a), or a segment of said second peptide, fused to either a DNA binding domain or its corresponding transcriptional activation

30 domain, whichever one is not employed in (a);

c) a reporter gene operatively associated with the transcriptional activation protein, or a portion thereof.

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d) optionally, a deletion or mutation in the chromosomal DNA of the yeast host cell for the

transcriptional activation protein if present in the host cell.

These novel modified host cells of the present invention can be used to determine the interaction of a test sample with a selected peptide of a peptide binding pair; e.g. the cell can be used to determine the interaction of a test sample with selected ligand or receptor.

A second aspect of the present invention relates to novel modified cells and screening methods which indicate the interaction of a test sample with a selected peptide and receptor by a recognizable change in phenotype. The cell exhibits the change in phenotype only in the presence of test compound having binding affinity for a peptide of the peptide binding pair, e.g. binding affinity for a ligand or its receptor.

A third aspect of the present invention relates to novel cells and screening methods which permit determining to which peptide of a peptide binding pair a test sample binds.

A fourth aspect of the present invention relates to novel cells which express three or more heterologous components for the study of higher order multi-protein associations between three or more peptides (e.g. such as the study of ligand dependent dimerization).

Defined Terms:

The term peptide binding pair refers to any pair of peptides having a known binding affinity for which the DNA sequence is known or can be deduced. The

peptides of the peptide binding pair must exhibit preferential binding for each other over any components of the modified cell.

5 The term **peptide** as used in the above summary and herein means any peptide, polypeptide or protein, unless stated otherwise. as noted above the peptides of a peptide binding pair can be a ligand and its corresponding receptor, or a ligand and any peptide
10 having a known binding affinity for the ligand.

15 **Heterologous** as used in the above summary and herein means peptides which (1) are not expressed by the naturally-occurring host cell or (2) are expressed by the modified host cell by an expression method other than the expression method by which the host cell would normally express the peptide.

20 Unless specified otherwise, the term **receptor** as used herein encompasses the terms receptor, soluble receptor, transducer and binding protein. In preferred embodiments of the invention, the receptor employed is a receptor or soluble receptor, with receptor being more preferred.
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30 **Receptor**, as used herein means plasma membrane proteins that bind specific molecules, such as growth factors, hormones or neurotransmitters, and then transmits a signal to the cells' interior that causes a cell to respond in a specific manner. This includes single transmembrane proteins.

35 **Soluble receptor** means non-transmembrane form of a receptor which is able to bind ligand. These are receptors released from a cell either by proteolysis or by alternatively spliced mRNA.

Binding protein means proteins that demonstrate binding affinity for specific ligand. Binding proteins may be produced from separate and distinct genes. For a given ligand, the binding proteins that are produced from specific genes are distinct from the ligand binding domain of the receptor, or its soluble receptor.

Transducer means a molecule that allows the conversion of one kind of signal into another, and the molecule is readily known as a transducer for one or more the peptides of a peptide binding pair, e.g. a transducer for a ligand/receptor group.

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Brief description of the Drawings

Figure 1. is a schematic diagram of a cell which expresses from separate plasmids two heterologous fused proteins (one being the ligand fused to the activation domain of a transcriptional activation protein and the other fused protein being a receptor fused to the DNA binding domain of the transcriptional activation protein). The Figure shows the expression of the two fused proteins and the binding of the ligand and receptor, which brings together the binding domain and activation domain, reconstituting the transcriptional activation protein. Once the transcriptional activation protein is reconstituted and anchored by the DNA binding domain to the Upstream Activation Sequences (UAS) site, transcription of the reporter gene (HIS3) is initiated.

Figure 2. contains photographs of plates which show the results of the growth experiments conducted in example 1 for the stains CY722, CY723, CY724, and CY781 on non-selective medium and selective medium, photographs

A and B, respectively.

Figure 3. is a schematic diagram of a dimer model, in which the ligand binds to a dual receptor system. The schematic diagram depicts a cell which expresses proteins from three separate plasmids. Two heterologous fused proteins (one fused protein being a first receptor fused to the activation domain of a transcriptional activation protein and the other fused protein being a second receptor fused to the DNA binding domain of the transcriptional activation protein) are expressed and free ligand (i.e. ligand which is not fused to either of the two domains of the transcriptional activation protein) is expressed from a third plasmid. The figure shows the expression of the two fused proteins and the binding of the free ligand and two receptor fusions which brings together the binding domain and activation domain, reconstituting the transcriptional activation protein. Once the transcriptional activation protein is reconstituted and anchored by the DNA binding domain to the Upstream Activation Sequences (UAS) site, transcription of the reporter gene (HIS3) is initiated.

Figure 4. is a photograph of the growth plate obtained for the strains CY846 and CY847 from Example 3, showing ligand-dependent stimulation of receptor dimerization.

Detailed Description of the Invention

The modified cell of this invention employs a host cell. An effective host cell for use in the present invention simply requires that it is defined genetically in order to engineer the appropriate expression of heterologous fused proteins, reporter(s) and any other desired genetic manipulations. The host cell can be any eukaryotic cell, vertebrate or non-vertebrate. The host

cell can be mammalian as well as amphibian, e.g. a Xenopus egg cell. Preferably, the host cell is a fungal cell, e.g. Aspergilla or Neuropora. In more preferred embodiments the host cell is a yeast cell. In 5 alternatively preferred embodiments the yeast host cell is Saccharomyces cerevisiae, Schizosaccharomyces pombe or Pichia pastoris.

The modified host cell employs at least two 10 genes for expressing separately the two heterologous fusion proteins. One of these fusion proteins comprises a first peptide of a peptide binding pair, or segment of said first peptide, which is joined to either a DNA binding domain or its corresponding transcriptional 15 activation domain of a transcriptional activation protein. A second fusion protein comprising the second peptide of the peptide binding pair, or a segment thereof. The second peptide is fused to either a DNA binding domain or its corresponding transcriptional 20 activation domain, whichever one is not employed in the first heterologous fused protein. The activity of the binding between the peptides of the peptide binding pairs is monitored by the use of a reporter gene, which is 25 operatively associated with the transcriptional activation protein employed in the two fusion proteins.

The transcriptional activation protein can vary widely as long as the DNA binding domains and the activation domains are known or can be deduced by 30 available scientific methods. The transcriptional activation protein can be any protein having two components, a DNA binding component and an activation component, wherein the transcriptional activation protein contains an acidic alpha helix for the activation of 35 transcription. Preferably, the transcriptional activation protein is selected from Gal4, Gcn4, Hap1,

Adrl, Swi5, Ste12, Mcml, Yap1, Acel, Ppr1, Arg81, Lac9, Qal1F, VP16, LexA, non-mammalian nuclear receptors (e.g.ecdysone) or mammalian nuclear receptors (e.g. estrogen, androgens, glucocorticoids, mineralocorticoids, 5 retinoic acid and progesterone ; see also Picard et al., 1990). Preferably, the transcriptional activation protein is a yeast protein, and more preferably, the transcriptional yeast protein is selected from Gal4, Gcn4 or Adrl. It is noted that any DNA binding protein can be used which functions with an activation domain. A DNA binding protein can be substituted for the DNA binding domain of a transcriptional activation protein if the recognition sequences operatively associated with the reporter gene are correspondingly engineered. 10 Illustrative of non-yeast DNA binding proteins are mammalian steroid receptors and bacterial LexA (see Wilson et al., 1990)

The reporter gene is generally selected in 20 order that the binding of the domains of the transcriptional activation protein can be monitored by well-known and straightforward techniques. Preferably, the reporter gene is selected based on its cost, ease of measuring its activity and low background (i.e. the 25 activity can be determined at relatively low levels of expression of the reporter gene because of a high signal to background ratio and/or a relatively low or no uninduced activity). The reporter can be any reporter for which its activity can be detected by any means 30 available. Illustrative of reporters which can be used in the present invention are reporter genes selected from the group of:

- a) lacZ, Luciferase gene, green fluorescent protein gene, CAT
- b) genes complementing auxotrophies, such as HIS, URA, LEU, ARG, MET, ADE, LYS, TRP.

c) gene conferring antibiotic resistance, such as neo^r, KAN,

d) genes conferring sensitivity to a chemical such as CYH2, CAN1 (canavine resistance). In many 5 embodiments it may be convenient for the reporter gene to prevent growth (CYH2). Preferably, the activity of the reporter gene is indicated by colorimetric or fluorescent methods and/or by measuring growth of the yeast cell.

10 As noted previously, the peptide employed in the modified cell is a peptide of a peptide binding pair for which the DNA sequence is known as well as the sequence of the second peptide of the binding pair. The peptides can also be peptides of a peptide binding 15 complex which contains two or more peptides which bind each other to form the binding complex. The peptides of the peptide binding pair can be a specific ligand and a corresponding receptor or any other peptides which bind to each other preferentially, such subunits of an enzyme.

20 One of the significant advantages of this invention is the discovery that the modified cell employing the DNA binding and activation domains of a transcriptional protein can be used to monitor the 25 binding of peptides of a peptide binding pair which bind through extracellular interaction. Certainly, if desired peptides which bind through intracellular interaction can also be employed in any of the novel modified cells and 30 methods of this invention. The peptide can be from a mammalian cell or non-mammalian cell. One of the most important embodiments of the present invention relates to the application of the novel modified cells and 35 corresponding screening methods of this invention for studying numerous mammalian peptide interactions. The mammalian peptides include mammalian ligand/receptor interactions, such as hormone/receptor interactions.

Illustrative of peptide hormones which can be used in the present invention are peptides selected from, but not limited to, one of the following groups: (a) the group consisting of cytokines, interleukins, hematopoietic growth factors, insulin, insulin-like growth factors, growth hormone, prolactin, interferons, and growth factors; (b) ligands for G-protein coupled receptors; (c) ligands for nonvertebrate receptors; (d) ligands for guanylyl cyclase receptors;

5 (e) ligands for tyrosine phosphatase receptors.

In alternative embodiments the peptide is a growth factor is selected from epidermal GF, nerve GF, leukemia inhibitory factor, fibroblast GF, platelet-derived GF, vascular endothelial GF, tumor necrosis factor, oncostatin M, ciliary neurotrophic factor, erythropoietin, steel factor, placental lactogen and transforming GF β .

20 In various preferred embodiments the peptide hormone is a ligand for a G-protein coupled receptor, such as growth hormone releasing factor, secretin, vasoactive inhibitory peptide, glucagon, thyrotropin, interleukin-8, luteinizing hormone (LH) and follicle 25 stimulating hormone (FSH).

In additional alternative embodiments the peptide employed is a nonvertebrate peptide, such as those selected from the group consisting of plant systemin and insect differentiation peptides. However, in preferred embodiments the peptide is selected from the group consisting of mammalian peptides, and more preferably, mammalian peptide hormones.

30 35 It is also noted that specific types of receptors may also be a peptide of a peptide binding pair

or peptide binding complex. Illustrative of various receptors are those selected from one of the following groups: (a) a cell adhesion molecule; (b) an immunomodulatory, antigen recognition or presentation molecule or other related peptides. Illustrative of cell adhesion molecules are ICAM, VCAM, ECAM, fibronectin, integrin, selectin and fibrinogen. Illustrative of an immunomodulatory, antigen recognition or presentation molecule are T cell receptor complex, B cell receptor complex, Fc receptors, major histocompatibility complex I, major histocompatibility complex II, CD4, CD8, CD27, CD30, MAC complex.

It is also noted that specific types of transducers may also be used a peptide of a peptide binding pair or peptide binding complex. The transducer proteins employed can be any transducer protein which binds at least one of the peptides of the peptide binding pair or peptide binding complex. Transducer proteins include gp130, kh97, AIC2A, AIC2B.

Preferably, the heterologous fused proteins are expressed by transformation of the yeast cell with an autonomously-replicating plasmid capable of expressing the fusion protein although they can be expressed by chromosomal modification.

As noted, the screening methods of this invention are designed in order to detect the ability of a test sample to affect the binding of a peptide binding pair, e.g. ligand-receptor interaction. Basically, the method comprises determining the activity of the reporter gene upon adding a test sample to a modified host cell of the present invention under conditions suitable to detect the activity in the presence of a sample or under a condition for which the modified host cell exhibits such

activity only in the presence of a sample having binding interaction with the peptide binding pair. Preferably, the activity of the reporter gene is determined by measuring a change in selected phenotype which is directly correlated to activity of the reporter.

The novel modified cells of this invention are readily applied in various screening methods for determining the binding ability of a test sample. The test sample may be a peptide, which is preferably about two amino acids in length, or a non-peptide chemical compound. The non-peptide test sample includes compounds, complexes and salts as well as natural product samples, such as plant extracts and materials obtained from fermentation broths. The modified host cells are cultured under suitable conditions for growth to study the interaction of a test sample on the binding interaction of the peptide binding pair. The modified host cells are placed in a growth medium, which preferably contains agar, with the test sample applied to the surface of the growth medium. The growth medium is preferably a conventional liquid medium of growth reagents and water, such as yeast synthetic medium (YSM available from BIO101 (also see Rose et al., Methods in Yeast Genetics, 1990).

One of the embodiments of the present invention is directed to a novel modified host cell and screening method which indicate the interaction of a test compound with a selected peptide binding pair by a recognizable change in phenotype. This modified host cell exhibits the change in phenotype only in the presence of test compound having binding affinity for one of the peptides of the peptide binding pair. This host cell is referred to herein as a "rescue" system. Normally, a cell response is exhibited when the two domains of the

transcriptional activation protein interact. However, in a rescue system a positive indication of change in the phenotype does not occur when the two domains of the transcriptional activation protein interact. A positive 5 indication of change in the phenotype occurs only when a test sample interrupts the interaction of the two domains of the transcriptional activation protein. In a rescue system, a modified host cell is capable of expressing at least two heterologous fusion proteins. Further, the 10 host cell comprises: a reporter gene operatively associated with the transcriptional activation protein; wherein said reporter gene prevents the exhibition of a specific phenotype on a selective medium due to the expression of the transcriptional activation protein, or 15 a portion thereof. A mutation in the chromosomal DNA of the host cell allows for reversal of the detectable phenotype, on the selective medium, in the absence of expression of the reporter gene. If needed, there is a deletion or mutation in the chromosomal DNA of the host 20 cell for a transcriptional activation protein in order that transcriptional activation only occurs upon productive interaction of the selected binding pair. Only when a test sample interrupts the interaction of the two domains of the transcriptional activation protein 25 will the modified cell grow or survive, or exhibit another selected phenotype. Preferably, the phenotype corresponds to the growth of the cell.

Once a screening method, as discussed above, is 30 used to determine whether a test sample interacts with, or rather disrupts, the peptide binding observed in the absence of a test sample, a secondary screen is employed to determine the specific binding affinity of the test sample, i.e. to which peptide of the peptide binding pair 35 the test sample binds. The secondary screens employ the novel cells of this invention wherein cells are adapted

to exhibit a phenotype, or phenotypic change only in the presence of a test sample which binds one peptide of the peptide binding pair. One of the preferred methods for determining the specific binding characteristics of the test sample involves employing cells which contain an effective (relatively high) copy number of either fusion protein containing one of the peptides. An effective copy number is any copy number sufficient to enable determination of the specific binding of the test sample.

5 Preferably, the gene copy number is at least about 5, and preferably ranges from about 5 to about 50, with the higher copy numbers being the most preferred. The other fusion protein is maintained at a relatively low (1 to about 2 copies per cell) by either integration into a

10 cell chromosome or by utilizing chromosomal centromeric sequences on the expression plasmid. If a high copy number of a first peptide is used in a cell of this invention, the cell will be more sensitive to the presence of the test sample which binds the second peptide of the peptide binding pair since the limiting amount of second peptide determines the level of the activity of the reporter gene (i.e. the change in phenotype observed). Conversely, if a high copy number of a gene encoding the second peptide of a peptide

15 binding pair is used, the cell will be more sensitive to the presence of a test sample which binds the first peptide since the limiting amount of the second peptide determines the level of the activity of the reporter gene (i.e. the change in phenotype observed). A direct

20 comparison of effects of a test compound on the phenotypes of the two strains (receptor>>>ligand versus ligand>>>receptor) demonstrates the specific protein interaction of the compound. As discussed supra, the genes expressing the peptides as well as the reporter

25 gene are preferably expressed by transformation of the yeast cell with an autonomously-replicating plasmid.

An additional modified host cell of this invention is directed to cells which can be used to study peptides ligands which employ dual receptors or a receptor and a transducer for activation or transmission of a signal from the binding of multiple peptide binding components, i.e. three or more peptide binding components.

Receptor dimerization is a critical first step for signal transduction for certain classes of receptors. Dimer receptor structures can be composed of identical receptor units (examples: insulin receptor, IGF-I receptor, PDGF receptor, kinase inert domain receptor (KDR), or colony stimulating factor (CSF)-I receptor) or non-identical receptor units (example: IL-6R + gp130; insulin-IGF-I hybrid receptor; LIF + gp130; CNTF+gp130; various interferon receptors).

The components of a modified host cell for monitoring the binding activity of a peptide having "dual receptor" system are as follows: the gene sequence (a) is a gene sequence encoding a heterologous fusion protein; said fusion protein comprising one peptide of a multiple peptide binding complex, or segment of said peptide, which is joined to either a DNA binding domain or its corresponding transcriptional activation domain of a transcriptional activation protein; and the gene sequence (b) is a gene sequence encoding a heterologous fusion protein; said fusion protein comprising a second peptide of said multiple peptide binding complex, or a segment of said receptor, fused to either a DNA binding domain or its corresponding transcriptional activation domain, whichever one is not employed in (a). The modified host cell for studying a multiple peptide binding complex, such as a dual receptor system, also comprises an appropriate reporter gene and chromosomal mutations for

specific analysis of the peptide (ligand/receptor) interaction as discussed infra. One can express a third peptide (e.g. a ligand) to establish a control for comparative or competitive testing.

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As noted above, for the study of multiple binding peptide complexes, i.e. higher-order proteins which contain three or more peptides, one can actually use the modified host cell of the present invention to express three or more peptides. In the case of a tripeptide binding complex, any two of the peptides can be fused to the two components of the transcriptional activation protein. For example, to study the interaction of a ligand which interacts via receptor dimerization, one can express the receptors as fused proteins with the ligand being expressed as a nonfusion protein. This host cell system can be also be applied in studying multiprotein enzyme complexes. For any multipeptide binding complex, one can identify novel peptides which interact with the complex by expressing novel proteins from random complementary DNA sequences (e.g. a cDNA library) fused to one of the domains of a transcriptional activation protein. In such a system, one of the known peptides of the peptide binding complex is fused to the other domain of the transcriptional activation protein while other units of the peptide binding complex are expressed as nonfusion peptides. It is further noted that the number of peptides expressed by the modified host cell should only be limited by the available detection means and the capacity of the host cell.

The novel screening methods can be utilized to identify compounds interacting with any peptide binding pair, e.g. any receptor and/or ligand. Also, this modified cell system with a reporter gene to create a

screen can be applied to any protein-protein interaction to discover novel compounds that disrupt that interaction. As specific examples: a) protein kinases implicated in cancers can be inserted into the system to rapidly screen for novel compounds that block the kinase-target interaction and thus may serve as unique cancer therapeutics; b) viral coat proteins, such as human immunodeficiency virus glycoproteins, and corresponding cell surface receptor proteins, such as CD4, can be inserted into the system to rapidly screen for compounds that disrupt this interaction, and may serve as anti-viral agents; c) the two subunits for the Plasmodium ribonucleotide reductase enzyme can be expressed in the system to screen for compounds which prevent this specific protein association and thus may serve as novel anti-malarial agents.

The following Examples are provided to further illustrate various aspects of the present invention. They are not to be construed as limiting the invention.

Example 1: Specific and Reversible Ligand - Receptor Interaction

Genes encoding fusion proteins are generated by cloning growth hormone (GH) and growth hormone receptor (GHR) cDNA sequences into plasmids containing the coding region for the domains of Gal4. DNA binding domain (Gal4) fusions are constructed in pAS2, which is described in Wade Harper et al. Gene activation domain (Gal4) fusions are constructed in pACT-II, which is identical to pACT (described in Durfee et al., 1993) except with a modification of the polylinker region. Into the Bgl II site is added the following sequence: Bgl II - Hemagglutinin epitope - NdeI - NcoI - SmaI - BamHI - EcoRI - XhoI - Bgl II, as adapted from the polylinker sequence of pAS2 (Wade Harper et al., 1993). The cDNA

encoding the mature peptide for porcine GH is generated using standard polymerase chain reaction (PCR) techniques (see Finney, 1993).

5 Oligonucleotides prepared on an ABI oligosynthesizer are designed according to the published cDNA sequence for pig GH (see Su and El-Gewely, 1988). A 30 base 5' oligonucleotide contains a NcoI site (5'-CATGCCATGGAGGCCTTCCCAGCCATGCC 3') and a 27 base 3' oligonucleotide contains a BamHI site (5'-CGGGATCCGCAACTAGAAGGCACAGCT-3'). The GH cDNA is generated using a pig pituitary lambda gt11 library as template source. A 540 bp fragment is obtained, ligated into pCR II vector (Invitrogen Corp.), recombinants are confirmed by restriction enzyme digest, and the DNA produced as described in Maniatus et al., 1982. The cDNA sequence is confirmed by dye-deoxy terminator reaction using reagents and protocols from Perkin-Elmer Cetus Corp. and an ABI 373A automated sequencer. The GH cDNA is directionally cloned into pACT-II via NcoI and BamHI sites. The cDNA encoding the extracellular domain of the GHR is generated using standard PCR methods. A 33 base 5' oligonucleotide containing a NcoI site (5'-CATGCCATGGAGATGTTCTGGAAAGTGGGGCT-3') and a 39 base 3' oligonucleotide containing a termination codon, followed by a NcoI site (5' -CATGCCATGGCCTACCGGAAATCTTCTTCACATGCTGCC-3') are used to generate a 742 bp fragment encoding amino acids 1-247 of the rat GHR (Baumbach et al., 1989). This GHR cDNA is cloned into pCRII vector as previously described above, and then subcloned into the NcoI site of the pAS2 vector. DNA of final recombinant vectors is transformed into yeast strain(s) by the lithium acetate method (Rose et al., 1990).

reporter gene is prepared according to the procedure described in Wade Harper et al., 1993. The genotype of strain Y190 is MAT_a leu2-3,112 ura3-52 trp1-901 his3d200 ade2-101 gal4 gal80 URA3::GAL-lacZ LYS2::GAL-HIS3 cyh^r.

5 Strain Y190 is transformed with both fusion constructs or with a single fusion construct plus the opposing vector containing no heterologous sequences. All strains are found to exhibit equal growth on nonselective medium (Figure 2A). These strains are then tested for growth on

10 selective medium (i.e. a growth medium lacking an amino acid which is synthesized by activation of the reporter gene). Only the strain containing both hybrid proteins (CY722) is able to grow while the strains containing either the ligand or receptor fusion alone do not grow

15 (CY724 and CY723, respectively; Figure 2B). Two independent samples of each strain are streaked on synthetic medium containing 2% glucose, yeast nitrogen base, ammonium sulfate, 0.1 mM adenine and 60 mM 3-amino-triazole (plate B) or on the same medium supplemented

20 with histidine (plate A). Plate A is incubated at 30 C for three days; plate B for five days. These results demonstrate that GH and GHR can mediate the Gal4-dependent activation of the reporter gene in an interaction suggestive of ligand - receptor binding.

25

Example 1A: Competing Expressed Free Ligand (GH) in the Presence of GH and GHR Fusion Proteins.

To substantiate the apparent binding of GH to its receptor in the foreign environment of a yeast nucleus, the system is modified to add a third plasmid mediating expression of "free" ligand to show that the GH peptide competes with the GH-Gal4 fusion protein, reversing the 2-hybrid interaction shown in Example 1. The parental strain Y190 (Wade Harper et al., 1993) is grown on a medium containing 5-fluoro-orotic acid to select for derivatives that spontaneously lose the URA3 gene (see

Rose et al., 1990). The resultant strain, designated CY770, is utilized for all experiments examining effects of protein expressed concurrently from the third component (i.e. third plasmid). The cDNA encoding GH is 5 generated by PCR methods using a 38 base 5' oligonucleotide containing an EcoRI site (5'-CCGAATTCAAAATGGCCTTCCCAGCCATGCCCTTGTCC-3') and a 26 base 3' oligonucleotide containing a HindIII site (5'CCAAGCTTCAACTAGAAGGCACAGCT-3') for subsequent 10 subcloning into the vector pCUP. pCUP is an inducible yeast expression vector derived from pRS316 (Hill et al., 1986). Briefly, this vector is constructed by inserting the 3' end of the yeast PGK gene (from pPGK; Kang et al., 1990) into the pRS316 cloning region as a BamHI-SalI 15 fragment to serve as a transcriptional terminator. To this plasmid, the CUP1 promoter region (Butt et al., 1984) is amplified by PCR as a SacI-EcoRI fragment and inserted into corresponding sites of the plasmid to create pCUP. The GH expression plasmid (GH-pCUP) is then 20 co-transformed with the GH and GHR fusion constructs into strain CY770 to generate CY781. Concurrent expression of free GH with the GH and GHR fusion proteins (CY781) is shown to block GH-GHR-dependent cell growth on selective medium (Figure 2B). This experiment typifies 25 an in vivo competition assay and demonstrates the reversibility of the observed ligand-receptor interaction.

Example 1B: Binding of Peptide Hormone Prolactin (PRL)
30 and its Receptor

To expand and validate this technology, a similar system was developed using the peptide hormone prolactin (PRL) and its receptor. Prolactin is structurally related to GH and the prolactin receptor (PRLR) is also 35 a member of the cytokine receptor superfamily. Unlike human GH, sub-primate GH does not readily bind the PRLR

(Young and Bazer, 1989); nor does PRL readily bind the GHR (Leung et al., 1987). Mature porcine PRL is generated as a fusion to the GAL4 activation domain. Oligonucleotides are designed to pig PRL (obtained from Genbank X14068), and used to generate the mature pig PRL protein hormone from a pig pituitary lambda gt11 library using standard PCR methods. A 31 base 5' oligonucleotide includes an EcoRI site (5' - CGGAATTCTGCCATCTGCCAGCGGGCCT-3') and corresponds to sequences encoding amino acids 1-7. A 30 base 3' oligonucleotide contains an EcoRI site (5' - GAATTCACGTGGGCTTAGCAGTTGCTGTCG-3') and corresponds to a region of cDNA 3' to the endogenous termination codon. A 600 bp fragment is obtained, ligated into pCR II vector, and confirmed by restriction enzyme digest and sequence analysis. The PRL cDNA is cloned into pACT-II via the EcoRI site.

The extracellular domain of the porcine PRL receptor (PRLR) is generated as a fusion to GAL4 DNA binding domain. Oligonucleotides are designed based on sequence of the mouse PRLR (Davis and Linzer, 1989). A 31 base 5' oligonucleotide contains a SmaI site (5' - TCCCCCGGGATGTCATCTGCACTTGCTTAC-3') while the 31 base 3' oligonucleotide contains a termination codon followed by a SalI site (5' TCCGTCGACGGTCTTCAGGTGAAGTCATT-3'). These oligonucleotides flank the extracellular domain of the PRLR, encoding amino acids 1-229. A pig pituitary lambda gt11 library is used as template source. Using standard PCR methods, a 687 bp fragment is generated, ligated into pCRII, and the nucleotide sequence is confirmed. The PRLR cDNA is cloned into the pAS2 vector via the SmaI and SalI restriction sites.

Strain Y190 was transformed with the PRL or PRLR fusion expression plasmids either alone (CY727 or CY728,

respectively) or together (CY726). Cells expressing both the PRL and PRLR fusions are able to grow on selective medium while the strains containing either the ligand or receptor fusion alone can not. These results mirror 5 those observed in the GH-GHR system in the examples above and establish the general utility of the 2-hybrid system for examination of ligand binding to members of this receptor superfamily.

10 Example 1C: Additional Confirmation of Ligand-Receptor Specificity for the Novel Yeast Host Cell System

Additional strains are developed to assess ligand-receptor specificity. URA minus strains 15 expressing GH and GHR fusion proteins are transformed with pCUP or PRL-pCUP; while strains expressing PRL and PRLR fusion proteins are transformed with pCUP, or PRL-pCUP. Briefly, PRL-pCUP is constructed in a fashion similar to that described for GH-pCUP. The PRL cDNA is 20 generated by PCR using a 33 base 5' oligonucleotide with an EcoRI site (5'-GAATTCAAAATGCTGCCCATCTGCCCGAGCGGG-3') and the 3' oligonucleotide in example 1B. The resulting fragment is introduced into pCUP via the EcoRI site. As demonstrated in the above Examples, a strain expressing 25 the GH and GHR fusions with no competitor grows on selective medium and this growth is abolished with coexpression of free GH. The prolactin experiment produces similar results which confirm the specificity of the ligand-receptor binding in the yeast cell. A 30 strain carrying PRL and PRLR fusions (CY787) can grow on selective medium and this growth is abrogated by expression of free PRL (CY786; Table 1).

To test selectivity of the GHR, a strain containing the 35 GH and GHR fusions is transformed with PRL-pCUP. This strain grows on selective medium (CY785; Table 1). These

data indicate that GH binding to its receptor in this system can be efficiently competed by excess GH (CY751) binding but not by the related PRL peptide (CY755). The results from the above experiments, expressing three 5 heterologous proteins, illustrates the specificity of ligand-receptor interaction(s) in the system of this invention.

TABLE 1. Strain list and bioassay results.

	Designation	AD fusion	BD fusion	pCUP	Growth
	CY700	-	-	-	0
5	CY722	GH	GHR	-	+
	CY723	vector	GHR	-	0
	CY724	GH	vector	-	0
	CY726	PRL	PRLR	-	+
	CY770	-	-	-	0
10	CY781	GH	GHR	GH	0
	CY784	GH	GHR	vector	+
	CY785	GH	GHR	PRL	+
	CY786	PRL	PRLR	PRL	0
	CY787	PRL	PRLR	vector	+

15

All yeast strains are derived from strain Y190 (Wade Harper et al. 1993). The genotype is MATA gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL-lacZ LYS2::GAL-HIS3 cyh. Strains with number designations equal to or greater than 770 do not have the URA3::GAL-lacZ gene. A dash indicates that a strain does not contain the denoted plasmid.

20

AD fusions are pACT derivatives; GH or PRL fused to the Gal4 activation domain.

BD fusions are pAS2 derivatives; extracellular domains of GH or PRL receptors fused to the DNA binding domain of Gal4.

pCUP denotes peptides expressed from the pCUP plasmid.

25

Summary of bioassay results. Each strain is grown on selective medium for 3 to 5 days at 30C then scored for cell growth, indicated by a plus.

EXAMPLE 2: Screen for compounds disrupting ligand-receptor interaction.

5 Low-copy-number plasmids expressing GHR- or GH-Gal4 fusion proteins (pOZ153 and pOZ152, respectively) are constructed to reduce expression of these proteins. In addition, a novel reporter gene is constructed that prevents cell proliferation on selective medium unless expression is abrogated. To construct the GHR fusion expression plasmid, a SacI-BamHI restriction fragment containing a yeast constitutive promoter and GAL4 sequences is isolated from pAS1 (Durfee et al., 1993) and cloned into pUN30 (Elledge and Davis, 1988). The 10 extracellular domain of GHR is then fused to GAL4 by ligation as an NcoI fragment as described in Example 1 to create pOZ153. To construct the GH fusion expression construct the entire GH-Gal4 region with promoter and terminator sequences is isolated from the 15 plasmid described in Example 1 as a PvuI-SallI fragment. This DNA segment is cloned into pUN100 (Elledge and Davis, 1988) generating pOZ152. A reporter gene is constructed by isolating the yeast CYH2 coding region and operatively linking it to a GAL promoter in a yeast 20 expression plasmid. Briefly, the GAL1 promoter region is inserted into YEp352 (Hill et al., 1986) as a 685 bp EcoRI-BamHI fragment. CYH2 sequences are amplified by PCR using oligonucleotides primers (5'-GGATCCAATCAAGAATGCCTTCCAGAT-3' and 5'-GCATGCGTCATAGAAATAATACAG-3') and pAS2 as the template. 25 The PCR product is digested with BamHI plus SphI and cloned into the corresponding sites in the YEp352-GAL vector. These plasmids are transformed into yeast strain CY770 which carries a mutation at the 30 chromosomal cyh2 gene rendering the strain resistant to the protein synthesis inhibitor cycloheximide. The 35

presence of all three plasmids is necessary to confer cycloheximide sensitivity (cyh⁻).

The strain (CY857) containing the ligand and receptor fusion plasmids plus the reporter plasmid forms the basis of a simple primary screen for compounds that disrupt the binding of GH to its receptor. Strain CY857 is embedded in standard yeast growth medium containing 10.0 µg/ml cycloheximide. Due to the ligand/receptor interaction driving expression of the CYH2 reporter gene, the strain is cyh⁻ and thus unable to grow. Chemical compounds are placed on this test medium. Compounds which impair GH-GHR binding are identified by the growth of cells surrounding the compound because in the absence of CYH2 expression the cells become resistant to cycloheximide present in the medium.

Secondary Screen to Determine Target of Sample

Disruption of ligand-receptor binding in this assay can result from reaction of the compound with either the receptor or ligand fusion component. The specific target of the novel compound is determined by a simple secondary assay utilizing strains overexpressing one of the fusion proteins. Strain CY858 expresses the GHR-GAL4 fusion in large excess due to the construct being maintained within the cells at high copy number (pOZ149), while the GH-fusion (pOZ152) is maintained at levels similar to the base strain (CY857). Conversely, strain CY859 expresses the GH-GAL4 fusion in large excess due to this construct being maintained within the cells at high copy number (pKY14), while the GHR fusion (pOZ153) is maintained at levels similar to base strain (CY857). Compounds rescuing growth in the primary screen using CY857 (GH

and GHR fusions expressed on low copy numbers plasmids) are then assayed in the same manner using CY858 (GHR>>GH) or CY859 (GH>>GHR) as the test strain. For example, when ligand - receptor binding is inhibited by
5 a compound reacting with the GHR, the secondary screen will demonstrate a detectable change for the phenotype measured. Secondary testing of the rescuing compound on strain CY858 which overexpresses the GHR fusion produces a smaller growth in the presence of the
10 compound than that observed for CY859. This detectable change in the measured phenotype occurs because the overabundance of GHR titrates the compound thereby increasing CYH2 expression and inhibiting cell growth. CY859 produces a detectable change similar to CY857
15 because the GHR fusion protein is limiting. A compound interacting with the ligand fusion demonstrates the inverse change in measured phenotype in this secondary assay.

20

Example 3: Demonstration of ligand dependent receptor dimerization:

25 Multiple protein interactions (for example; ligand - receptor - receptor) are investigated with the expanded system which expresses a third protein using the following scheme.

30 One unit of the receptor dimer is generated as a fusion protein with either the Gal 4 DNA binding or activation domain. The other unit of the receptor dimer is generated as a fusion protein with corresponding Gal DNA binding or activation domain, whichever is not used for the first fusion. The gene encoding the ligand is
35 expressed from the third plasmid and is produced as a free (non-fusion) ligand. Interaction of the fusion

proteins occurs only in the presence of ligand (see Figure 3).

The interaction of vascular endothelial cell growth factor (VEGF) with the ligand binding domain of its cognate receptor (KDR, kinase insert domain containing receptor) is described as an example for this system. KDR is a tyrosine kinase receptor, and dimer formation (1 ligand - 2 receptors) is suggested to be important for hormone-induced receptor function. The cDNA encoding the ligand domain of KDR (Terman et al., 1991) is isolated as an Nco I - BamHI fragment and cloned into both the pACT-II and pAS2 vectors. The cDNA encoding the mature protein for VEGF is generated using standard PCR techniques. Oligonucleotides are designed from published sequence (see Tischer et al., 1991). A 34 base 5' oligonucleotide containing an EcoRI site (5'-CGGAATTCGAAGTATGGCACCCATGGCAGAAGGA-3') and a 28 base 3' oligonucleotide containing an EcoRI site (5'-CGGAATTCTGGATCCTCATTCATTCA-3') are used to generate a 450 bp fragment encoding the mature protein and cloned into the EcoRI site of pCUP. DNA of final recombinant vectors is transformed into yeast by the lithium acetate method to generate appropriate strains.

25

The yeast host strain (CY770) is transformed with KDR-pACT-II, KDR-pAS2 and VEGF-pCUP to generate strain CY846; or transformed with both receptor fusions and pCUP to generate strain CY847.

30

Additionally, both KDR-pACT-II and KDR-pAS2 are transformed together (CY845) or separately (CY843 or CY844) or VEGF-pCUP alone (CY841) as control strains. Strains are tested for growth on selective medium. The strain (CY846) that expresses the VEGF ligand plus the two receptor fusion proteins exhibits substantial growth on selective media in comparison to the strain

CY847, which does not express the VEGF ligand (see Figure 4). These results demonstrate that the effective cells of this invention can be used to study ligand-dependent dimerization of the receptor.

5

Example 4: Screen for compounds that act as ligands in a dimer receptor system

10

Dimerization (oligomerization) of receptor units is often an important first step in activation of receptors such as those for the growth factors, cytokines and those described supra. The novel cell system described in example 3 can be applied to the discovery of novel compounds which promote (or block) receptor dimerization. Such novel interacting compounds may serve as effective therapeutic agents for pathologies associated with these receptors.

20

Plasmids expressing the dimer receptor unit(s) as fusion proteins are generated as discussed in example 3. The strain (CY845) containing the KDR-pACT-II and KDR-pAS2 fusions serves as an example of a simple primary screen for receptors which exhibit a dimer structure. Strain CY845 is embedded in synthetic agar medium deficient in histidine (Rose et al., 1990). Test compounds are applied to the top of this test medium. Chemical compounds which induce interaction of the two receptor fusions (in the absence of ligand) results in the reconstitution of the endogenous transcriptional activator, which is linked to a reporter gene, such as HIS3. The reconstitution is identified by growth of cells surrounding the compound.

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What We Claim Is:

1. A modified host cell which comprises:

a) a gene sequence encoding a heterologous fusion protein; said fusion protein comprising a first peptide of a peptide binding pair, or segment of said first peptide, which is joined to either a DNA binding domain or its corresponding transcriptional activation domain of a transcriptional activation protein;

b) a gene sequence encoding a heterologous fusion protein, said fusion protein comprising a second peptide of the peptide binding pair in (a), or a segment thereof, fused to either a DNA binding domain or its corresponding transcriptional activation domain, whichever one is not employed in (a);

c) a reporter gene operatively associated with the transcriptional activation protein, or a portion thereof;

d) optionally, a deletion or mutation in the chromosomal DNA of the host cell for the transcriptional activation protein if present in the selected host cell;

wherein said modified host is capable of expressing at least two heterologous fusion proteins and at least one reporter gene.

2. The modified host cell of claim 1 wherein said host cell is a eukaryotic cell.

3. The modified host cell of claim 1 wherein said host cell is a mammalian or amphibian cell.

4. The modified host cell of claim 1 wherein said host cell is a fungal cell.
5. The modified host cell of claim 4 wherein said fungal host cell is a Aspergilla or Neuropora.
6. The modified host cell of claim 4 wherein said fungal cell is a yeast cell.
7. The modified host cell of claim 6 wherein said yeast cell is selected from Saccharomyces cerevisiae, Schizosaccharomyces pombe or Pichia pastoris.
8. The modified host cell of claim 1 wherein the peptides of the peptide binding pair interact with each other via extracellular activity in their natural environment.
9. The modified host cell of claim 1 wherein the peptides of the peptide binding pair interact with each other via intracellular activity in their natural environment.
10. The modified host cell of claim 1 wherein the peptide binding pair is selected from the group consisting of mammalian peptides.
11. The modified host cell of claim 1 wherein the peptide binding pair is selected from the group consisting of non-mammalian peptides.
12. The modified host cell of claim 1 wherein the peptide binding pair is selected from the group consisting of ligands and a corresponding receptor.
13. The modified host cell of claim 1 wherein one

peptide of the peptide binding pair is a hormone.

14. The modified host cell of claim 13 wherein one peptide of the peptide binding pair is selected from the group consisting of mammalian peptides hormones.

15. The modified host cell of claim 1 wherein said transcriptional activation protein is selected from (a) Gal4, Gcn4, Hap1, Adrl, Swi5, Ste12, Mcml, Yap1, Ace1, Ppr1, Arg81, Lac9, QalF, VP16, LexA or (b) mammalian nuclear receptors.

16. The modified host cell of claim 15 wherein said transcriptional activation protein is a yeast transcriptional activation protein.

17. The modified host cell of claim 16 wherein said transcriptional activation yeast protein is GAL4 or lac9.

18. The modified host cell of claim 1 wherein the reporter gene is selected from the group of:

- a) lacZ, Luciferase gene, green fluorescent protein gene, chloron phenicol acetyl transferase;
- b) genes complementing auxotrophies
- c) genes conferring antibiotic resistance; and
- d) genes conferring sensitivity to a chemical.

19. The modified host cell of claim 1 wherein at least one of the heterologous fused proteins are expressed by transformation of the yeast cell with an autonomously-replicating plasmid capable of expressing said fusion protein.

20. The modified host cell of claim 19 wherein the

heterologous fused proteins are expressed by transformation of the yeast cell with an autonomously-replicating plasmid capable of expressing said fusion protein.

21. The modified host cell of claim 19 wherein at least one peptide of the peptide binding pair is selected from one of the following groups:

- (a) the group consisting of cytokines, interleukins, hematopoietic growth factors, insulin, insulin-like growth factors, growth hormone, prolactin, interferons, and growth factors;
- (b) ligands for G-protein coupled receptors;
- (c) ligands for nonvertebrate receptors;
- (d) ligands for guanylyl cyclase receptors;
- (e) ligands for tyrosine phosphatase receptors;

22. The modified host cell of claim 21 wherein the peptide is a growth factor selected from epidermal GF, nerve GF, leukemia inhibitory factor, fibroblast GF, platelet-derived GF, vascular endothelial GF, tumor necrosis factor, oncostatin M, ciliary neurotrophic factor, erythropoietin, steel factor, placental lactogen and TGF.

23. The modified host cell of claim 21 wherein at least one peptide of the peptide binding pair is a transducer.

24. The modified host cell of claim 23 wherein the transducer is selected from the group of transducer proteins gp130, kh97, AIC2A and AIC2B.

25. The modified host cell of claim 1 wherein at least one peptide of the peptide binding pair is a receptor is selected from one of the following groups:

- (a) a cell adhesion molecule; and
- (b) an immunomodulatory, antigen recognition or presentation molecule.

26. The modified host cell of claim 25 wherein the receptor is a cell adhesion molecule selected from ICAM, VCAM, ECAM, fibronectin, integrin, selectin and fibrinogen.

27. The modified host cell of claim 25 wherein the receptor is an immunomodulatory, antigen recognition or presentation molecule selected from T cell receptor complex, B cell receptor complex, Fc receptors, major histocompatibility complex 1, major histocompatibility complex 2, CD4, CD8, CD27, CD30, MAC complex.

28. The modified host cell of claim 21 wherein one peptide of the peptide binding pair is a ligand for G-protein coupled receptors.

29. The modified host cell of claim 28 wherein the ligand for G-protein coupled receptors is selected from growth hormone releasing factor, secretin, vasoactive inhibitory peptide, glucagon, interleukin-8, luteinizing hormone (LH) follicle stimulating hormone (FSH) and thyrotropin.

30. The modified host cell of claim 11 wherein the nonvertebrate peptides is selected from the group

consisting of plant systemin and insect differentiation peptides.

31. A method of detecting the ability of a test sample to affect the binding interaction of a peptide binding pair; said method comprising:

determining the activity of the reporter gene upon adding a test sample to the modified host cell of claim 1 under conditions suitable to detect said activity in the presence of said sample or under a condition for which the modified host cell exhibits said activity only in presence a sample having binding interaction of a peptide binding pair.

32. The method of claim 31 wherein the activity of the reporter gene is determined by measuring a selected phenotype which is dependent on the activity of the reporter.

33. The modified host cell of claim 1 wherein the repoter gene (c) is operatively associated with the transcriptional activation yeast protein; wherein said reporter gene, on a selective medium, prevents the exhibition of a detectable phenotype in the presence of expression of the transcriptional activation yeast protein, or a portion thereof; and wherein the host cell further comprises:

(e) optionally, a deletion or mutation in the chromosomal DNA of the host cell allowing for exhibition of the selected phenotype on the selected medium in the absence of expression of the reporter gene of plasmid (c), if needed for the reporter gene (c) to be the only source of measured activity.

34. A method of detecting the ability of a test sample

to affect interaction of a peptide binding pair; said method comprising:

observing a change in the measured phenotype of a modified host of claim of claim 33 upon adding a test sample to said modified host cell; said cell being cultured under conditions suitable to detect a selected phenotype in the presence of a test sample or cultured under a condition for which said cell exhibits the phenotype only in the presence of a test sample having interaction with the peptide binding pair.

35. A method of determining whether a test sample, which exhibits interaction with a peptide of a peptide binding pair; said method comprising:

(a) adding a test sample to a modified host cell and comparing the exhibition of a selected phenotype in the presence and absence of the test sample;

wherein said modified host cell is adapted to exhibit a change in phenotype only in the presence of a test sample which binds to only one peptide of the peptide binding pair.

36. The method of claim 35 wherein said method employs a modified host cell which comprises:

a) a gene sequence encoding a heterologous fusion protein; said fusion protein comprising a first peptide of a peptide binding pair, or segment of said first peptide, which is joined to either a DNA binding domain or its corresponding transcriptional activation domain of a transcriptional activation protein; wherein the gene encoding said first peptide is present in an

effective copy number.

b) a gene sequence encoding a heterologous fusion protein, said fusion protein comprising a second peptide of the peptide binding pair in (a), or a segment of said second peptide, fused to either a DNA binding domain or its corresponding transcriptional activation domain, whichever one is not employed in (a);

c) a reporter gene operatively associated with the transcriptional activation yeast protein, or a portion thereof.

d) a deletion or mutation in the chromosomal DNA of the yeast host cell for the transcriptional activation protein.

37. The method of claim 36 wherein at least one of the heterologous fused proteins is expressed by transformation of the modified host cell with an autonomously-replicating plasmid capable of expressing said fusion protein.

38. The method of claim 36 wherein the reporter gene is expressed by transformation of the modified host cell with a plasmid capable of expressing said reporter gene.

39. The modified host cell of claim 1 wherein the gene sequence (a) is a gene sequence encoding a heterologous fusion protein; said fusion protein comprising one of two receptor molecules for a peptide, or segment of said receptor, which is joined to either a DNA binding domain or its corresponding transcriptional activation domain of a transcriptional

activation protein; and the gene sequence (b) is a gene sequence encoding a heterologous fusion protein; said fusion protein comprising the other receptor for the peptide, or a segment of said receptor, fused to either a DNA binding domain or its corresponding transcriptional activation domain, whichever one is not employed in (a);

40. The modified host cell of claim 39 wherein the gene in (a) encodes a heterologous fusion protein; said fusion protein comprising a transducer for a peptide, or segment of said transducer, which is joined to either a DNA binding domain or its corresponding transcriptional activation domain of a transcriptional activation protein.

41. The modified host cell of claim 40 wherein the transducer protein is selected from gp130, KH97, AIC2A, and AIC2B.

42. A method of detecting the ability of a test sample to function as a ligand for a receptor comprising:

determining the level of activity of the reporter gene of the modified host cell of claim 40 upon adding a test sample to said cell under conditions suitable to said activity.

43. The modified host cell of claim 1 wherein the gene sequence (a) is a gene sequence encoding a heterologous fusion protein; said fusion protein comprising one peptide of a multiple peptide binding complex, or segment of said peptide, which is joined to either a DNA binding domain or its corresponding

transcriptional activation domain of a transcriptional activation protein; and the gene sequence (b) is a gene sequence encoding a heterologous fusion protein; said fusion protein comprising a second peptide of said multiple peptide binding complex, or a segment of said receptor, fused to either a DNA binding domain or its corresponding transcriptional activation domain, whichever one is not employed in (a).

44. A method of detecting the ability of a test sample to function as a peptide component of a multiple peptide binding complex; said method comprising:

determining the level of activity of the reporter gene of the modified host cell of claim 43 upon adding a test sample to said cell under conditions suitable to detect said activity.

45. The modified host cell of claim 1 wherein the gene sequence (a) is a gene sequence encoding a heterologous fusion protein; said fusion protein comprising one peptide of a multiple peptide binding complex, or segment of said peptide, which is joined to either a DNA binding domain or its corresponding transcriptional activation domain of a transcriptional activation protein; and the gene sequence (b) is a gene sequence encoding a heterologous fusion protein; said fusion protein comprising a second peptide of said multiple peptide binding complex, or a segment of said receptor, fused to either a DNA binding domain or its corresponding transcriptional activation domain, whichever one is not employed in (a); wherein said modified host cell further comprises as (e) at least one gene sequence encoding a peptide; wherein said modified host is capable of expressing said peptides.

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46. The modified host cell of claim 45 wherein at least one of components (a), (b) or (e) is a gene sequence corresponding to a complementary DNA library.

47. The modified host cell of claim 46 wherein (b) is a gene sequence corresponding to a complementary DNA library.

48. A method of determining the ability of a random protein to function as a component of a multiple peptide binding complex; said method comprising:

determining the level of activity of the reporter gene of the modified host cell of claim 47 upon expression of the random protein from the DNA library under conditions suitable to detect said activity.

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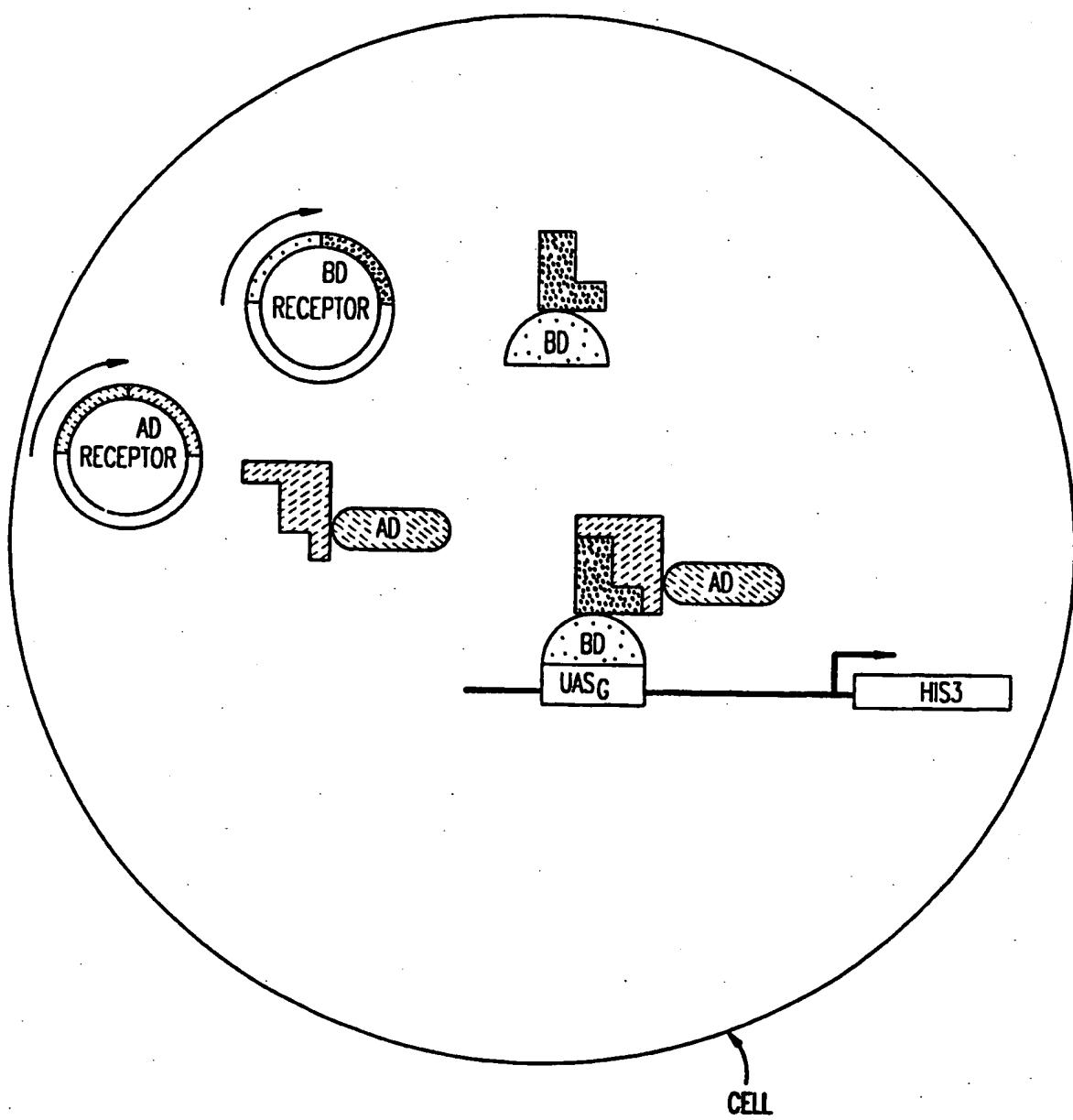


FIG.1

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CY723
GHR^{BD}



CY724
GHAD

CY723
GHR BD



CY724
GHAD

CY722
GHR^{BD} + GHAD

CY781
GHAD

CY722
GHR^{BD} + GHAD

CY781
CHR BD + GHAD + GH

FIG. 2B

FIG. 2A

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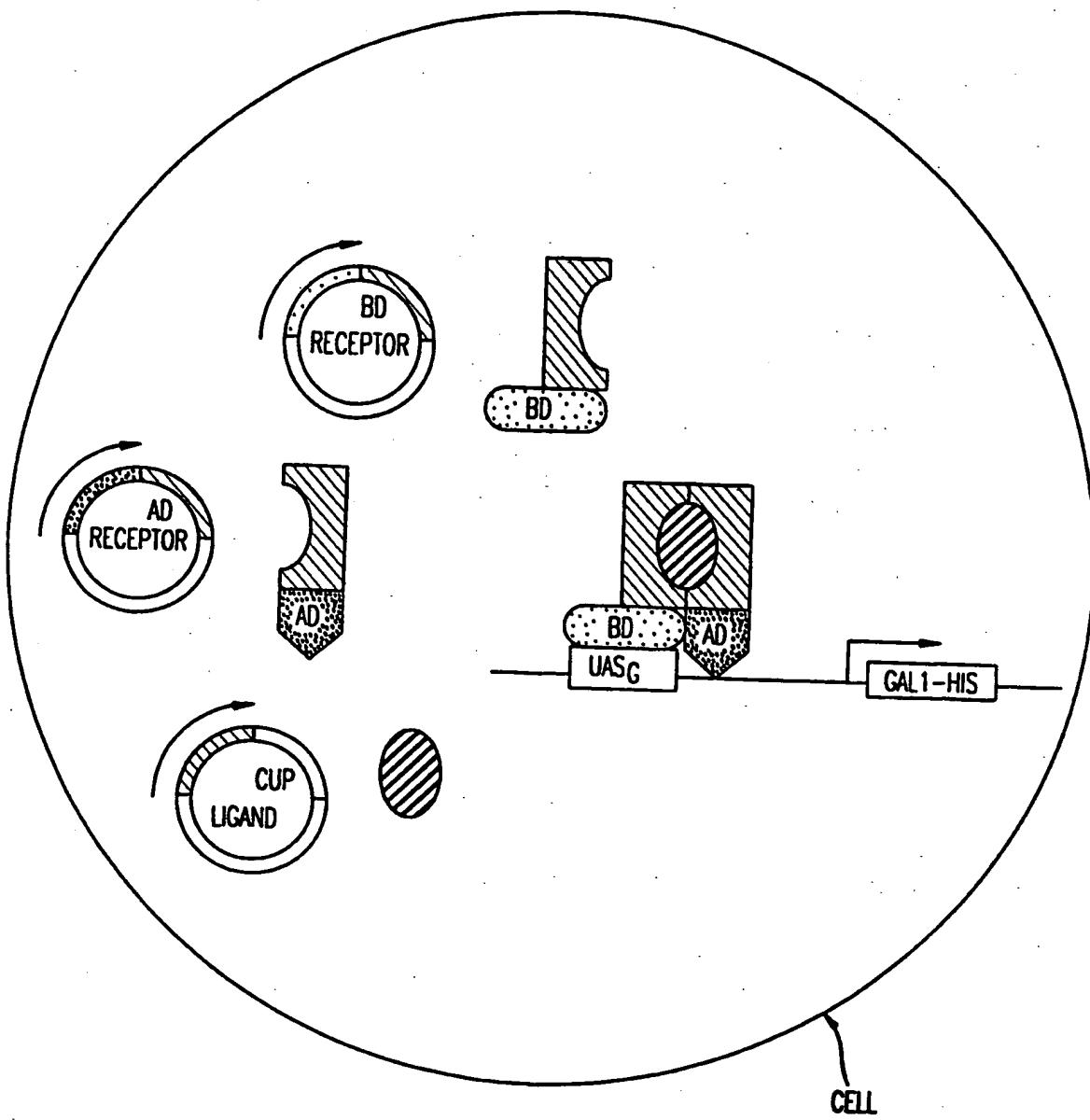


FIG.3

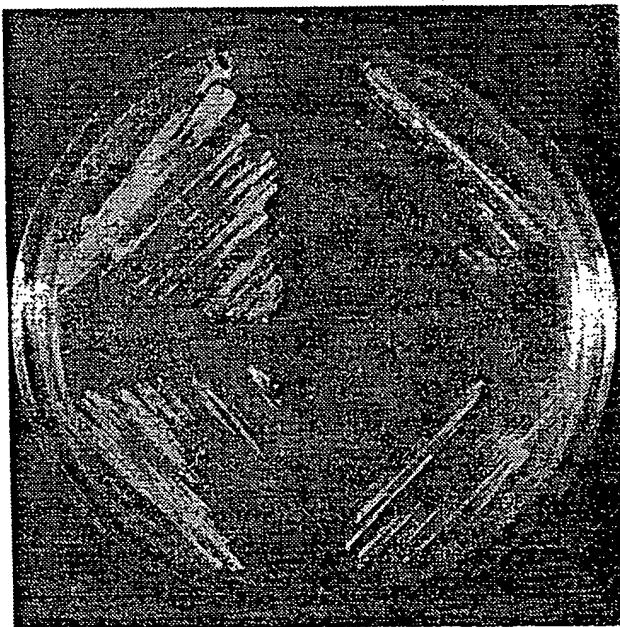
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CY846
KDR^{BD}
KDR^{AD}
VEGF-pCUP



CY847
KDR^{BD}
KDR^{AD}
pCUP

FIG. 4

INTERNATIONAL SEARCH REPORT

Intern. Application No.
PCT/US 95/06895

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/00 C12N1/19 C12N15/18 C12Q1/68 C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 88, November 1991 WASHINGTON US, pages 9578-9582, C-T.CHIEN ET AL. 'The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest' cited in the application see page 9581 ---	1,2,4,6, 7,9,11, 15-20, 31-40, 42-48
X	SCIENCE, vol. 257, 31 July 1992 LANCASTER, PA US, pages 680-682, X.YANG ET AL. 'A protein kinase substrate identified by the two-hybrid system' cited in the application see the whole document ----	1,2,4,6, 7,9,11, 15-20, 31-40, 42-48

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

20 September 1995

Date of mailing of the international search report

13.10.95

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Cupido, M

INTERNATIONAL SEARCH REPORT

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C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO,A,94 09133 (THE GENERAL HOSPITAL CORPORATION) 28 April 1994 see page 1, line 14 - page 5, line 2 ---	1,8-15, 18-20, 31-40, 42-48
X	CELL, vol. 74, 16 July 1993 NA US, pages 205-214, A.VOJTEK ET AL. 'Mammalian Ras interacts directly with the serine/threonine kinase Raf' see page 206, left column, paragraph 3 ---	1,2,4, 6-15, 18-20, 31-40, 42-48
E	WO,A,95 18380 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 6 July 1995 see claims 1-19 ---	1-7,9, 10, 12-20, 31-40, 42-48
E	WO,A,95 19988 (ICOS CORPORATION) 27 July 1995 see claims 1-9,18 -----	1,2,4,6, 7,9,11, 15-20, 31-40, 42-48

INTERNATIONAL SEARCH REPORTIntern: Application No
PCT/US 95/06895

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		EP-A- 0665884	09-08-95		
WO-A-9518380	06-07-95	AU-B- 1436695	17-07-95		
WO-A-9519988	27-07-95	NONE			